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I, JONNE YABSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PS 2930 for a patent by WOMEN'S AND CHILDREN'S HOSPITAL as filed on 14 June 2002.

WITNESS my hand this
Twenty-fourth day of June 2003

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AUSTRALIA

PATENTS ACT 1990

PROVISIONAL SPECIFICATION FOR AN INVENTION ENTITLED:-

"IDENTIFICATION OF OLIGOSACCHARIDES AND THEIR USE IN THE DIAGNOSIS AND EVALUATION OF MUCOPOLYSACCHARIDOSES AND OTHER RELATED DISORDERS"

This invention is described in the following statement:

FIELD OF THE INVENTION

This invention relates to mucopolysaccharidoses (MPS), in particular to the identification of oligosaccharide structures present in the tissues and body fluids of these patients which provide useful biochemical markers (biomarkers) for the diagnosis, characterisation and clinical management of MPS.

BACKGROUND

Lysosomal storage disorders (LSD) represent a group of over 40 distinct genetic diseases that generally affect young children and have a devastating impact on the child and the family. Affected individuals can present with a wide range of clinical symptoms depending upon the specific disorder and the particular genotype involved. Central nervous system dysfunction, from behavioural problems to severe mental retardation, is characteristic of many LSD. In a specific LSD group, the mucopolysaccharidoses (MPS), other symptoms may include skeletal abnormalities, organomegaly, corneal clouding and dysmorphic features [Neufeld and Meunzer, 1995]. In severe cases, the child requires constant medical management but often dies before adolescence. The significance of LSD to health care becomes obvious when the group incidence rate for LSD (1:5,000 births) is compared with well-known and intensively studied genetic disorders such as phenylketonuria (1:14,000) and cystic fibrosis (1:2,500).

The MPS, with an incidence of 1:22,000 births in Australia, represent a major group of LSD. There are six types. MPS I (Hurler or Scheie syndrome) results from a deficiency of α-L-iduronidase and leads to the lysosomal storage of the glycosaminoglycans dermatan sulphate and heparan sulphate. MPS II (Hunter syndrome) results from a deficiency of iduronate-2-sulphatase and leads to the same glycosaminoglycans stored in lysosomes as found with MPS I. MPS III (Sanfilippo syndrome) has four sub-types with all resulting in the storage of the one glycosaminogylcan, heparan sulphate. MPS IIIA, MPS IIIB, MPS IIIC and MPS IIID result from deficiencies of sulphamidase, α-N-acetylglucosaminidase, glucosamine acetyl-CoA: N-acetyltransferase and N-acetylglucosamine-6-sulphatase respectively. MPS IV(Morquio syndrome) has two sub-types, MPS IVA and IVB, both with

lysosomal storage of the glycosaminoglycan keratan sulphate that results from a deficiency of N-acetylgalactosamine-6-sulphatase and β-galactosidase respectively. MPS VI (Maroteaux Lamy syndrome) results from lysosomal storage of the glycosaminoglycan dermatan sulphate due to a deficiency of N-acetylgalactosamine-4-sulphatase. MPS VII (Sly syndrome) results from a deficiency of β-glucuronidase and the lysosomal storage of dermatan sulphate and heparan sulphate.

Over the past 20 years there has been considerable progress in the diagnosis of LSD. The development and introduction of chromatographic-based urine screens for MPS and oligosaccharidoses has facilitated screening of clinically selected patients for these disorders. As a consequence, the first stage of diagnosis following a clinical index of suspicion for MPS and certain oligosaccharidoses is now a urine screen which, if positive, is then followed by a more specific enzymatic analysis. However, while simple to perform, these screening methods are relatively labour-intensive and often require experience to accurately interpret results and consequently are not used as frequently in some centres. Furthermore, the fact that these screens are not amenable to automation has also restricted their use in other screening strategies such as newborn screening.

The production of specific substrates and antibody capture assays has made the enzymatic analyses for LSD very accurate and specific, although many of these assays are complex and require cultured cells or tissue biopsies, thus making them expensive, time-consuming and invasive. As a result, testing for LSD is often not a first line strategy and therefore diagnosis of an affected child can take months or years and often requires multiple visits to a range of specialists. This process is extremely stressful on the patient and family. There is therefore a need for the development of fast, accurate and economical screens for earlier diagnosis.

With the development of new therapies for many of these disorders, the diagnostic requirements for LSD are also changing. The efficacy of many of the proposed therapies will rely heavily upon early detection and treatment, before the onset of irreversible pathologies. Newborn screening for LSD promises to provide such early

detection; however, pre-symptomatic diagnosis raises a number of issues relating to patient management and treatment. Ideally, therapy should be commenced prior to the onset of pathology. However, the wide clinical spectrum displayed in many LSD will make selection of therapy options difficult unless additional information relating to disease progression can be determined. The establishment of dose levels required for either drug or enzyme replacement therapy (ERT) will be uncertain and therefore hazardous without some detail as to clinical severity. In addition, methods to monitor the effects of therapy in a clinically unaffected individual are required.

Except for those cases with a family history of the disease, pre-symptomatic detection of LSD can only be achieved by newborn screening. Currently, even after the presentation of clinical symptoms, the diagnosis of a LSD is a complex process involving a range of assays performed on urine, blood and, in some disorders, skin fibroblasts. These assays are time-consuming, expensive and invasive, making them unsuitable for newborn screening applications. Therefore there is a need to identify useful biomarkers for the LSD. These biomarkers have application in the development of a newborn screening program, as well as the potential to address a number of the other issues highlighted above.

One common feature of LSD is the accumulation of storage of material within lysosomes. This material is normally degraded within the lysosome, and the products of degradation are usually transported across the lysosomal membrane. It is generally recognised that the accumulation and storage of material results in an increase in the number and size of lysosomes within the cell from approximately 1% to as much as 50% of total cellular volume. The inventors have previously found that the level of certain lysosomal proteins would be elevated as a result of storage and that these proteins may prove to be useful biomarkers for the detection of all LSD [Meikle et al 1997; Hua et al 1998]. The lysosome-associated membrane proteins (LAMPs), the saposins and α–glucosidase have been identified as useful biomarkers and sensitive immunoquantification assays have been developed for these proteins. While LAMP-1 or LAMP-2 enables the identification of an 'at increased risk' group which contains up to 65% of LSD affected individuals, the inclusion of one of the

saposins will raise this figure up to approximately 85%.

A second characteristic feature of LSD is that the stored substrates are released from cells either by exocytosis or as a result of cell death. Consequently, the identification and quantification of these stored substrates in blood or urine can confirm a LSD and indicate which type of disorder is involved, which will be critical for the monitoring of therapy trials (animal and human) in the coming years.

In the MPS group of disorders, the primary storage material is made up of the glycosaminoglycans, in particular heparan sulphate, dermatan sulphate, keratan sulphate and chondroitin sulphate. As it is known that certain endoglycosidases are capable of cleaving these glycosaminoglycans, the inventors investigated the possibility that, in addition to biomarkers such as the glycosaminoglycans and larger fragments, there would be smaller oligosaccharide fragments that would be useful biomarkers for the MPS group of disorders.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers, but not the exclusion of any other element or integer or group of elements or integers.

SUMMARY OF THE INVENTION

The inventors have identified that elevated levels of certain oligosaccharides (in particular, sulphated oligosaccharides) in a biological sample taken from a subject are indicative of an increased likelihood that the subject has MPS or multiple sulphatase deficiency. The oligosaccharides of interest will generally comprise 2 to 6 sugar residues (i.e. they will range from disaccharides to hexasaccharides). These oligosaccharides are derived from the glycosaminoglycan storage substrates present in these disorders.

Accordingly, a method of screening for, or monitoring, a disorder, being a

mucopolysaccharidosis (MPS) or multiple sulphatase deficiency, in a mammal comprises: measuring the quantity of certain oligosaccharides (in particular, sulphated di- to hexa-saccharides) in a biological sample taken from the mammal, wherein said oligosaccharides are capable of formation by cleavage of one or more glycosaminoglycans (GAGS), and wherein elevated quantities of said oligosaccharides are indicative of the presence or extent of the disorder. The oligosaccharides may, for example, be derived from one or more of the following GAGS: heparan sulphate, dermatan sulphate, keratan sulphate and chondroitin sulphate. The method of the invention can be used, for example, for neonatal screening or for monitoring progress of a patient being treated for a MPS.

The oligosaccharides can, for example, be identified using tandem mass spectrometry, mass spectrometry, liquid chromatography and/or immunoassay. In one embodiment, they are identified using electrospray-ionisation tandem mass spectrometry (ESI/MSMS) and characterised using a combination of enzyme digestion and ESI/MSMS. The determination of these oligosaccharide markers by, for example, ESI/MSMS enables the identification of MPS affected subjects from the analysis of their body fluids or tissue samples such as urine, plasma, blood or skin fibroblasts. In addition, based on the experimental results and higher levels of different oligosaccharides that have been identified in the biological samples taken from subjects (see Table 1 on page 17), the inventors have further identified oligosaccharides which, at elevated levels, are indicative of a specific type of MPS disorder (see the following list). In the MPS VI group, some of the oligosaccharides have been identified and structure confirmed with experimental data, as a specific example of the invention.

In the following list, the following abbreviations apply:
UA = uronic acid; HNAc = N-acetylhexosamine; HN =hexosamine; Hex = hexose; (S) = sulphate (sugar residue not defined); IdoA = iduronic acid; GlcA = glucuronic acid; GalNAc = N-acetylgalactosamine.

MPS I

Dermatan sulphate fragments:

UA-HNAc(S)
UA-HNAc(Sx2)
UA-HNAc-UA
UA-HNAc-UA(S)
UA-HNAc–UA(Sx2)
UA-HNAc-UA(Sx3)
UA-HNAc-UA-HNAc
UA-HNAc-UA-HNAc(S)
UA-HNAc-UA-HNAc(Sx2)
UA-HNAc-UA-HNAc(Sx3)
UA-HNAc-UA-HNAc(Sx4)
UA-HNAc-UA-HNAc-UA(S)
UA-HNAc-UA-HNAc-UA(Sx2)
UA-HNAc-UA-HNAc-UA(Sx3)
UA-HNAc-UA-HNAc-UA(Sx4)
UA-HNAc-UA-HNAc-UA(Sx5)

Heparan sulphate fragments:

UA-HNAc
UA-HNAc(S)
UA-HNAc(Sx2)
UA-HNAc-UA
UA-HNAc-UA(S)
UA-HNAc-UA(Sx2)
UA-HNAc-UA(Sx3)
UA-HNAc-UA-HNAc
UA-HNAc-UA-HNAc(S)
UA-HNAc-UA-HNAc(Sx2)
UA-HNAc-UA-HNAc(Sx3)
UA-HNAc-UA-HNAc(Sx4)
UA-HNAc-UA-HNAc-UA(S)
UA-HNAc-UA-HNAc-UA(Sx2)
UA-HNAc-UA-HNAc-UA(Sx3)
UA-HNAc-UA-HNAc-UA(Sx4)
UA-HNAc-UA-HNAc-UA(Sx5)

UA-HN(S)
UA-HN(Sx2)
UA-HN-UA(S)
UA-HN-UA(Sx2)
UA-HN-UA(Sx3)
UA-HN-UA-HNAc(S)
UA-HN-UA-HNAc(Sx2)
UA-HN-UA-HNAc(Sx3)
UA-HN-UA-HNAc(Sx4)

UA-HN-UA-HNAc-UA(S)
UA-HN-UA-HNAc-UA(Sx2)
UA-HN-UA-HNAc-UA(Sx3)
UA-HN-UA-HNAc-UA(Sx4)
UA-HN-UA-HNAc-UA(Sx5)

UA-HNAc-UA-HN(S)
UA-HNAc-UA-HN(Sx2)
UA-HNAc-UA-HN(Sx3)
UA-HNAc-UA-HN(Sx4)
UA-HNAc-UA-HNAc-UA(S)
UA-HNAc-UA-HNAc-UA(Sx2)
UA-HNAc-UA-HNAc-UA(Sx3)
UA-HNAc-UA-HNAc-UA(Sx4)
UA-HNAc-UA-HNAc-UA(Sx5)

UA-HN-UA-HN-UA(S)
UA-HN-UA-HN-UA(Sx2)
UA-HN-UA-HN-UA(Sx3)
UA-HN-UA-HN-UA(Sx4)
UA-HN-UA-HN-UA(Sx5)
UA-HN-UA-HN-UA(Sx6)

MPS II

Same oligosaccharides as MPS I except an extra S on each

MPS IIIA

Heparan sulphate derived:
HN(S)
HN(Sx2)
HN-UA(S)
HN-UA(Sx2)
HN-UA(Sx3)
HN-UA-HNAc(Sx3)
HN-UA-HNAc(Sx4)
HN-UA-HNAc-UA(S)
HN-UA-HNAc-UA(Sx2)
HN-UA-HNAc-UA(Sx3)
HN-UA-HNAc-UA(Sx4)
HN-UA-HNAc-UA(Sx5)

HN-UA-HNAc(S)
HN-UA-HNAc(Sx2)
HN-UA-HNAc(Sx3)
HN-UA-HNAc(Sx4)
HN-UA-HNAc-UA(S)
HN-UA-HNAc-UA(Sx2)

HN-UA-HNAc-UA(Sx3)
HN-UA-HNAc-UA(Sx4)
HN-UA-HNAc-UA(Sx5)

HN-UA-HN(Sx2)
HN-UA-HN(Sx3)
HN-UA-HN(Sx4)

HN-UA-HN-UA(Sx2)
HN-UA-HN-UA(Sx3)
HN-UA-HN-UA(Sx4)
HN-UA-HN-UA(Sx5)
HN-UA-HN-UA(Sx6)

MPS IIIB
Heparan sulphate derived:
HNAc-UA
HNAc-UA(S)
HNAc-UA-HNAc
HNAc-UA-HNAc(S)
HNAc-UA-HNAc(Sx2)
HNAc-UA-HNAc(Sx3)
HNAc-UA-HNAc(Sx4)
HNAc-UA-HNAc-UA
HNAc-UA-HNAc-UA(S)
HNAc-UA-HNAc-UA(Sx2)
HNAc-UA-HNAc-UA(Sx3)
HNAc-UA-HNAc-UA(Sx4)

HNAc-UA-HN(S)
HNAc-UA-HN(Sx2)
HNAc-UA-HN(Sx3)
HNAc-UA-HN-UA(S)
HNAc-UA-HN-UA(Sx2)
HNAc-UA-HN-UA(Sx3)
HNAc-UA-HN-UA(Sx4)
HNAc-UA-HN-UA(Sx5)

MPS IIIC
Heparan sulphate derived:
HN-UA
HN-UA(S)
HN-UA-HNAc(Sx2)
HN-UA-HNAc(Sx3)
HN-UA-HNAc-UA(S)
HN-UA-HNAc-UA(Sx2)
HN-UA-HNAc-UA(Sx3)

HN-UA-HNAc-UA(Sx4)

HN-UA(Sx2)
HN-UA-HNAc(S)
HN-UA-HNAc-UA(S)
HN-UA-HNAc-UA(Sx2)
HN-UA-HNAc-UA(Sx3)
HN-UA-HNAc-UA(Sx4)

HN-UA-HN(S)
HN-UA-HN(Sx2)
HN-UA-HN(Sx3)

HN-UA-HN-UA(S)
HN-UA-HN-UA(Sx2)
HN-UA-HN-UA(Sx3)
HN-UA-HN-UA(Sx4)
HN-UA-HN-UA(Sx5)

MPS IIID
Heparan sulphate derived:
HN(S)
HNAc(S)
HN-UA(S)
HN-UA(Sx2)
HN-UA(Sx3)
HN-UA-HNAc(S)
HN-UA-HNAc(Sx2)
HN-UA-HNAc(Sx3)
HN-UA-HNAc(Sx4)
HN-UA-HNAc-UA(S)
HN-UA-HNAc-UA(Sx2)
HN-UA-HNAc-UA(Sx3)
HN-UA-HNAc-UA(Sx4)
HN-UA-HNAc-UA(Sx5)

HN-UA-HNAc(S)
HN-UA-HNAc(Sx2)
HN-UA-HNAc(Sx3)
HN-UA-HNAc(Sx4)
HN-UA-HNAc-UA(S)
HN-UA-HNAc-UA(Sx2)
HN-UA-HNAc-UA(Sx3)
HN-UA-HNAc-UA(Sx4)
HN-UA-HNAc-UA(Sx5)

HN-UA-HN(Sx2)

HN-UA-HN(Sx3)
HN-UA-HN(Sx4)

HN-UA-HN-UA(Sx2)
HN-UA-HN-UA(Sx3)
HN-UA-HN-UA(Sx4)
HN-UA-HN-UA(Sx5)
HN-UA-HN-UA(Sx6)

HNAc-UA(S)
HNAc-UA-HNAc(S)
HNAc-UA-HNAc(Sx2)
HNAc-UA-HNAc(Sx3)
HNAc-UA-HNAc(Sx4)
HNAc-UA-HNAc-UA(S)
HNAc-UA-HNAc-UA(Sx2)
HNAc-UA-HNAc-UA(Sx3)
HNAc-UA-HNAc-UA(Sx4)

HNAc-UA-HN(Sx2)
HNAc-UA-HN(Sx3)
HNAc-UA-HN-UA(Sx2)
HNAc-UA-HN-UA(Sx3)
HNAc-UA-HN-UA(Sx4)
HNAc-UA-HN-UA(Sx5)

MPS IVA
Keratan sulphate derived:
HNAc(S)
HNAc(Sx2)
HNAc-Hex(S)
HNAc-Hex(Sx2)
HNAc-Hex-HNAc(S)
HNAc-Hex-HNAc(Sx2)
HNAc-Hex-HNAc(Sx3)

Chondroitin sulphate derived:
HNAc-UA(S)
HNAc-UA(Sx2)
HNAc-UA-HNAc(S)
HNAc-UA-HNAc(Sx2)
HNAc-UA-HNAc(Sx3)
HNAc-UA-HNAc(Sx4)

HNAc-UA-HNAc-UA(S)
HNAc-UA-HNAc-UA(Sx2)
HNAc-UA-HNAc-UA(Sx3)

HNAc-UA-HNAc-UA(Sx4)

MPS IVB

Keratan sulphate derived:

Hex-HNAc(S)

Hex-HNAc-Hex(S)

Hex-HNAc-Hex(Sx2)

MPS VI

Dermatan sulphate derived:

HNAc-UA(S)

HNAc-UA(Sx2)

HNAc-UA-HNAc(S)

HNAc-UA-HNAc(Sx2)

HNAc-UA-HNAc(Sx3)

HNAc-UA-HNAc(Sx4)

HNAc-UA-HNAc-UA(S)

HNAc-UA-HNAc-UA(Sx2) Experiments using recombinant lysosomal enzymes to sequentially digest these oligosaccharide structures, in combination with mass spectroscopy of the digest at each step, produced data showing the non-reducing end UA in one oligosaccharide to be IdoA, and that another oligosaccharide, with the same mass, has GlcA in this position.

HNAc-UA-HNAc-UA(Sx3)

HNAc-UA-HNAc-UA(Sx4)

HNAc-UA-HNAc-UA-HNAc(S)

HNAc-UA-HNAc-UA-HNAc(Sx2)

HNAc-UA-HNAc-UA-HNAc(Sx3)

HNAc-UA-HNAc-UA-HNAc(Sx4)

HNAc-UA-HNAc-UA-HNAc(Sx5)

HNAc-UA-HNAc-UA-HNAc-UA(Sx4) Experiments using recombinant lysosomal enzymes to sequentially digest these oligosaccharide structures, in combination with mass spectroscopy of the digest at each step, produced data showing the sequence of the first two non-reducing end residues as N-acetylgalactosamine-4-sulphate-iduronic acid-2-sulphate (GalNAc4S-IdoA2S).

MPS VII

Dermatan sulphate derived:

UA-HNAc(S)

UA-HNAc(Sx2)

UA-HNAc-UA(S)

UA-HNAc-UA(Sx2)

UA-HNAc-UA(Sx3)

UA-HNAc-UA-HNAc(Sx3)

UA-HNAc-UA-HNAc(Sx4)
UA-HNAc-UA-HNAc-UA(S)
UA-HNAc-UA-HNAc-UA(Sx2)
UA-HNAc-UA-HNAc-UA(Sx3)
UA-HNAc-UA-HNAc-UA(Sx4)

Heparan sulphate derived:
UA-HNAc(S)
UA-HNAc(Sx2)
UA-HNAc-UA(Sx3)
UA-HNAc-UA-HNAc(Sx3)
UA-HNAc-UA-HNAc(Sx4)
UA-HNAc-UA-HNAc-UA(S)
UA-HNAc-UA-HNAc-UA(Sx2)
UA-HNAc-UA-HNAc-UA(Sx3)
UA-HNAc-UA-HNAc-UA(Sx4)
UA-HNAc-UA-HNAc-UA(Sx5)

This information can be useful in the prediction of disease progression in these patients and the monitoring of therapy.

A diagnostic kit for use in the method of the invention will comprise reagents (e.g. antibodies) capable of binding to one or more of the oligosaccharides of interest.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: msms spectra of a disaccharide found in MPS I urine.

Parent ion $[m/z]^{-1}$ = 806.2, representing a sulphated disaccharide isolated from MPS I urine, showing an array of daughter ions, including m/z 256 and 295 used for the MRM pairs (see Table 1 on page 17).

Figure 2: ms spectra showing characterisation of the non-reducing end of a trisaccharide and a tetrasaccharide isolated from MPS I urine.

A fraction from the Bio-Gel P2 column containing abundant amounts of trisaccharide and tetrasaccharide was subjected to enzymatic digestion with recombinant α-L-iduronidase. Spectra A show the tetrasaccharide $[m/z]^{-2}$ = 632.6 and a trisaccharide $[m/z]^{-1}$ = 982.4 and $[m/z]^{-2}$ = 490.9 in the absence of α-L-iduronidase. Spectra B show m/z of each of the ions 632.6, 982.4 and 490.9 following enzymatic digestion

with α-L-iduronidase after removal of iduronic acid, producing 544.4, 806.4 and 402.8 respectively.

Figure 3: Relative concentrations of oligosaccharides in urine from control and MPS I patients.

Urine samples (1 μmol creatinine equivalent) from controls and MPS I patients were derivatised and analysed on the tandem mass spectrometer. Relative quantification was achieved by reference to an internal standard (GlcNAc6S(d3)). Each plot represents a different oligosaccharide. N = number of samples in each group. Centre bars show the median value for each group, shaded areas show the 25th and 75th percentiles, and top and bottom bars show the limits of the range. o represents statistical outliers.

METHODS

Isolation of oligosaccharides from urine: Urine from a mucopolysaccharidosis type I (MPS I) patient was obtained with consent and 500ml was centrifuged at 2000xg for 10 min to remove debris. The clarified urine was then passed over a 50ml column of DEAE-Sephacel previously equilibrated with 0.1 M $NaCOOCH_3$ pH 5.0 under gravity. The column was then washed with 10 column volumes of the equilibration buffer and urinary glycosaminoglycans (GAGs) were eluted in 0.1 M $NaCOOCH_3$ pH 5.0 containing 1.2 M NaCl. Fractions were assayed for uronic acid and those containing substantial quantities were pooled, lyophilised and reconstituted in 4.0ml of water. The pooled GAGs were then fractionated on a Bio-Gel P2 column (1.5cm ID x 170cm length) previously equilibrated in 0.5 M NH_4COOH. Fifty-five fractions of 4.0ml were collected and assayed for uronic acid. Fractions 31 to 54 were subsequently derivatized for mass spectrometry as described below.

Preparation of deuterated N-acetylglucosamine-6-sulphate (GlcNAc6S(d3)): GlcNAc6S(d3) was prepared by selective N-acetylation of the glucosamine-6-sulphate (GlcN6S). The monosaccharide GlcN6S (25mg) was dissolved in anhydrous solutions of pyridine (700 μl), dimethyl formamide (700 μl) and methanol (50 μl) by sonication. The solution was stirred on ice and acetic anhydride(d_6) (2 x 20 μl) was

added at 30 minute intervals. After one hour, the reaction was quenched with a 4% aqueous ammonia solution (500μl) and then placed under a stream of nitrogen to remove solvents. This step was repeated. The remaining residue was dissolved in water (500 μl) and loaded onto an anion exchange column (AG1-X8, H^+ form, 100-200 mesh, 5 ml bed) and washed with deionised water (4 column volumes). The monosaccharide was eluted with LiCl (2.5 M) and the fractions monitored with the bicinchoninic acid microwell assay (BCA™, Pierce Chemical Company, Rockford Illinois). The fractions containing GlcNAc6S(d3) were pooled and de-salted on a size exclusion column (50 x 1 cm) of P2 fine (Bio-Rad). The desalted fractions were pooled and lyophilised to produce 5.5 mg of a white solid (18.7 % yield). The purity of the GlcNAc6S(d3) was determined by comparison to the undeuterated GlcNAc6S in the mass spectrometer. A neutral loss (NL374) ESI/MSMS experiment showed that the GlcNAc6S(d3) compound was 83.95 % pure (w/w).

Derivatisation of oligosaccharides: Samples from column fractions, urine and cultured skin fibroblast extracts were lyophilised prior to derivatisation. Whole blood samples were dried onto filter paper (S&S 903, Schleicher & Schuell, Dassel, Germany) and 3mm punches were taken and derivatised directly. Each blood spot sample, with 50 μl of derivatising solution (250 mmol/L 1-phenyl-3-methyl-5-pyrazolone (PMP), 400 mmol/L NH_3, pH 9.1) containing 100 pmol methyl lactose and 100 pmol GlcNAc6S(d3) added, was vortexed vigorously. Lyopholised column fractions were resuspended in 200 μl of derivatising solution, and cell extracts and urine samples (1 μmol creatinine equivalents) were resuspended in 100 μl of the same solution containing 1 nmol GlcNAc6S(d3). Samples were then heated in an oven at 70°C for 90 minutes. Samples were then acidified with a 2 fold molar excess of formic acid and made up to 500 μl with water. Each sample was extracted with 500 μl of $CHCl_3$ to remove excess PMP and centrifuged (13,000xg, 5 minutes). Solid phase extraction columns (25 mg, C18) were primed with successive 1 ml washes of 100% CH_3CN, 50% CH_3CN/0.025% formic acid and water. The aqueous layer from each $CHCl_3$ extraction (400 μl) was applied to a primed C18 column and allowed to enter the solid phase completely. The column was washed with water (1 X 500 μl, followed by 2 X 1000 μl) and dried under vacuum (15 minutes) on a Supelco,

Visiprep24 vacuum manifold (Sigma-Aldrich, St Louis, USA), or in a lyophiliser (45 minutes), if in the 96-well format. Each dried C18 column was then washed with $CHCl_3$ (2 X 1000 µl) to remove any unincorporated PMP, and again dried thoroughly. Derivatised oligosaccharides were eluted from each C18 column with 50% CH_3CN / 0.025% formic acid in water (3 X 200 µl). The derivatised blood spots were dried under a stream of N_2 and reconstituted in 100 µl of 50% CH_3CN / 0.025% formic acid in water for injection into the mass spectrometer. Column fractions, fibroblast extracts and urine sample eluates were injected directly into the mass spectrometer.

Characterisation of oligosaccharides: Oligosaccharides isolated from MPS I urine were characterised by their fragmentation patterns as assessed by tandem mass spectrometry. Although tandem mass spectrometry is used in this example, it is to be understood that other quantitive methods, including chromatographic methods, immunoassay and liquid chromatography-mass spectrometry, can also be used as quantitative methods to determine the quantity of oligosaccharides within a biological sample.

The subject may or may not have already been diagnosed with an MPS disorder. The clinical condition of the subject may be monitored to determine the efficacy of a treatment regime (e.g. enzyme replacement therapy, gene therapy and/or dietary therapy). Product ion scans (Figure 1) show daughter ions produced from the parent ion, yielding information about their structure as well as providing suitable ion pairs for multiple reaction monitoring (MRM). Furthermore, the nature of the sugar residues within a particular oligosaccharide can be further characterised by enzymatic cleavage with recombinant enzymes deficient in the particular MPS. An example given in Figure 2 identifies the non-reducing end of a trisaccharide and tetrasaccharide isolated from MPS I urine to be iduronic acid. A full list of oligosaccharides isolated and characterised is shown in Table 1.

Table 1 Oligosaccharide analytes identified in MPS Patients. Oligosaccharides were purified from urine from MPS patients and analysed by tandem mass spectrometry. Voltages were optimised and MRM ion pairs were identified to enable the rapid analysis of these oligosaccharide species in biological samples.

		Q1[2]	Q3[3]	Q3[4]	Negative ion voltages[5]						Source of
Analyte	Oligosaccharide[1]	*m/z*	*m/z*	*m/z*	DP set	FP set	CE	CE*	EP set	CXP set	Oligosaccharide[6]
1	UA	523.4	173.2		-31.0	-200.0	-64.0		10.0	-7.0	All MPS
2	HNAc	550.3	173.2		-31.0	-200.0	-64.0		10.0	-7.0	All MPS
3	HNAc(S)	630.4	256.1		-42.0	-200.0	-42.0		10.0	-15.0	All MPS
4	HNAc(Sx2)	710.0	256.0		-36.0	-160.0	-58.0		10.0	-17.0	All MPS
5	HNAc-UA	726.2	331.1	173.2	-31.0	-190.0	-28.0	-64.0	10.0	-7.0	All MPS
6	HNAcS-UA	806.0	331.0	173.2	-26.0	-210.0	-46.0	-82.0	10.0	-13.0	MPS VI
7	UA-HNAc(S)	806.0	295.0		-76.0	-290.0	-48.0		10.0	-7.0	MPS I
8	UA-HNAc-UA	901.8	727.2		-41.0	-190.0	-30.0		10.0	-15.0	MPSI/MPS II
9	UA-HNAc-UA(S)	982.4	331.1	269.0	-51.0	-240.0	-46.0	-78.0	10.0	-7.0	MPSI/MPS II
11	UA-HNAc-UA(Sx2)	1062.0	982.2		-51.0	-250.0	-28.0		10.0	-15.0	MPSI/MPS II
21	UA-HNAc-UA(Sx3)	1143.1	632.0		-101.0	-350.0	-58.0		10.0	-19.0	MPS II
22	UA-HNAc-UA(Sx4)	1223.7	632.7		-66.0	-330.0	-74.0		10.0	-21.0	MPS II
12	HNAc-UA-HNAc-UA	1105.8	931.4		-46.0	-270.0	-32.0		10.0	-11.0	MPS IIIB
13	HNAc-UA-HNAc-UA(S)	1185.0	1105.0		-51.0	-250.0	-28.0		10.0	-15.0	MPS IIIB
14	UA-HNAc-UA-GlcNAc(Sx2)	632.3	298.0		-46.0	-220.0	-38.0		10.0	-9.0	MPS I
15	HN(Sx2)	668.2	256.0		-41.0	-200.0	-42.0		10.0	-15.0	MPS VI
16	HN-UA	684.4	331.1	173.2	-31.0	-190.0	-40.0	-64.0	10.0	-7.0	All MPS
17	HNS-UA	764.4	331.1	173.2	-31.0	-190.0	-40.0	-64.0	10.0	-7.0	MPSIIIA
18	UA-HN-UA(S)	940.0	269.0		-91.0	-330.0	-76.0		10.0	-15.0	MPS I
19	UA-HN-UA(Sx2)	1020.3	940.3	269.0	-66.0	-350.0	-26.0	-70.0	10.0	-17.0	MPS I
	UA-HN-UA(Sx2)	509.7	434.7		-26.0	-100.0	-16.0		10.0	-5.0	MPS I
InStd	d3 GlcNAc-6S	633.4	259.1		-41.0	-200.0	-42.0		10.0	-15.0	Synthesised

[1] UA = uronic acid; HNAc = N-acetylhexosamine; HNAcS = sulphated N-acetylhexosamine; HN = hexosamine; HNS = sulphated hexosamine; GlcNAc = N-acetylglucosamine; (S) = sulphate (sugar residue not defined); d3 GlcNAc-6S = d3 N-acetylglucosamine-6-sulphate.

[2] m/z in quadrapole 1

[3] m/z in quadrapole 3

[4] m/z in quadrapole 3 for second product ion

[5] voltages used on the SCIEX API 3000 tandem mass spectrometer

[6] oligosaccharides were purified from urine from MPS patients

Diagnosis of the mucopolysaccharidoses: As an example of the discriminating power of these oligosaccharides, the relative level of six oligosaccharides in urine from 8 controls and 14 MPS I patients was determined. For 5 of the 6 oligosaccharides measured, there was no overlap between the control and the MPS populations (Figure 3).

Monitoring of therapy: As an example of monitoring the effects of therapy, urine samples were obtained from an MPS I patient prior to a bone marrow transplant (BMT) being performed and at 3 and 8 months after the transplant. Each urine sample was analysed for six oligosaccharides shown to be present in MPS I urine. In all of the oligosaccharides, a continual decrease in concentration towards the normal range was observed (Table 2).

Table 2. Relative oligosaccharide levels in a MPS I patient pre and post BMT

Oligosaccharide [1]	MRM pairs	Pre-BMT	Post-BMT (3 months)	Post-BMT (8 months)	Control Avg (n=8)	Control Std Dev
UA-HN-UA(Sx2)	509/422	2.31	0.63	0.59	0.05	0.03
HNAc(S)	630/256	192	165	139	68	52
UA-HNAc(S)	806/295	13.93	8.39	2.12	0.04	0.02
UA-HN-UA(S)	940/269	0.49	0.21	0.19	0.03	0.02
UA-HNAc-UA(S)	982/269	2.58	1.86	1.05	0.06	0.04
UA-HNAc-UA-HNAc(Sx2)	632/298	9.34	4.51	2.57	0.23	0.16

[1] UA = uronic acid; HN = hexosamine; S = sulphate; HNAc = N-acetylhexosamine

Determination of phenotype: Human skin fibroblasts from 2 normal controls and 3 MPS I patients were cultured for 6 weeks post-confluence in BME supplemented with 10 % FCS. Cells were then harvested and cell extracts prepared and subsequently analysed by mass spectrometry for an oligosaccharide identified in the MPS I urine (Table 3). A marked difference was observed in the relative levels of the oligosaccharide in the control and MPS I cell lines. A significant difference between the oligosaccharide level in the two severe patients and the intermediate patient was also evident, demonstrating a relationship between oligosaccharide level and patient severity.

Table 3 Relative levels of oligosaccharides in skin fibroblasts from control and MPS I patients.

Cell line	Disorder	UA-HN-UA(Sx2)[1]
SF 5344	Control	0.034
SF 5248	Control	0.030
SF 2662	MPS I (intermediate)	0.090
SF 5048	MPS I (severe)	0.379
SF 538	MPS I (severe)	0.387

[1] relative level of oligosaccharide/mg cell protein
UA = uronic acid; HN = hexosamine

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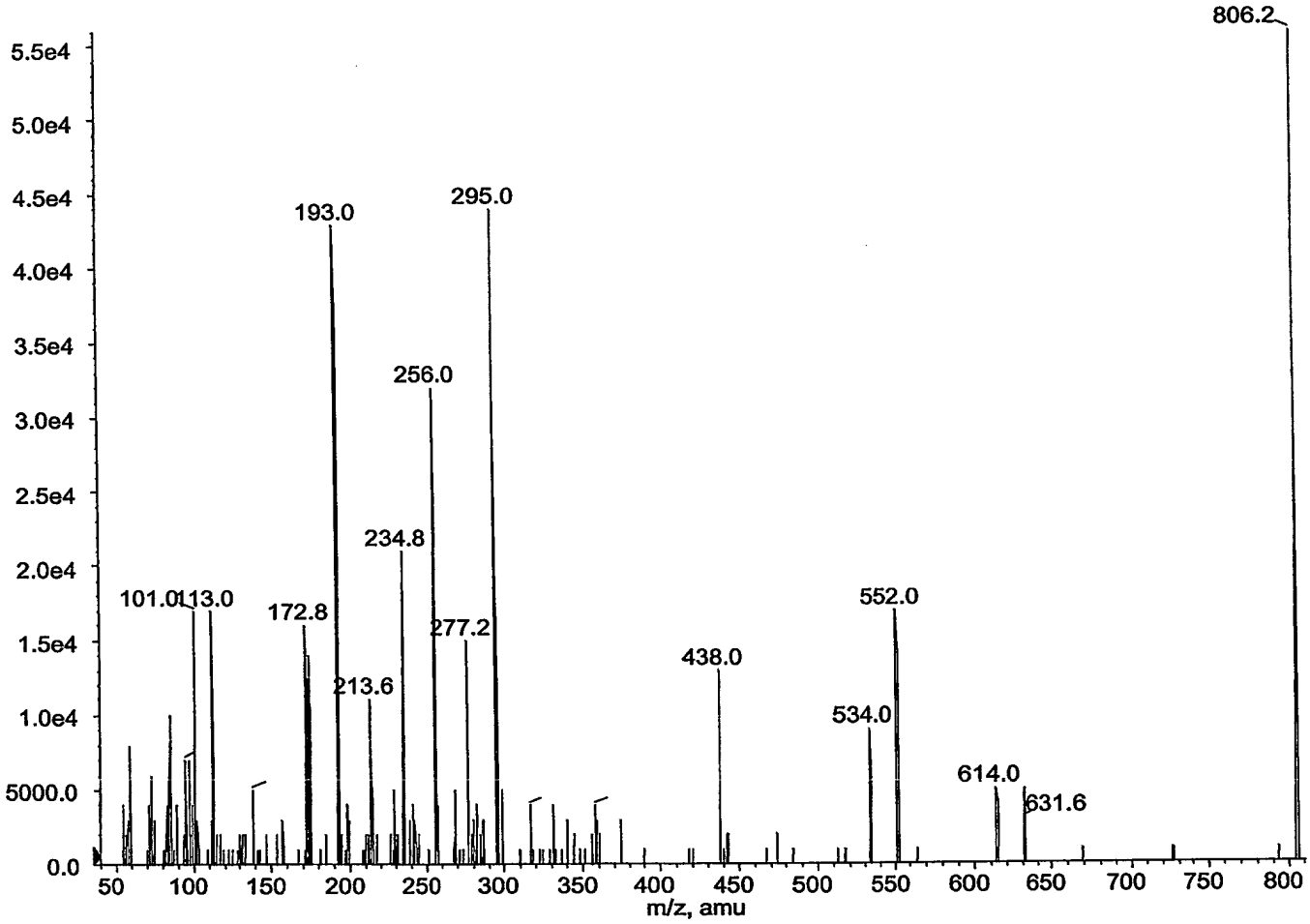
WOMEN'S AND CHILDREN'S HOSPITAL
By its Patent Attorneys
MADDERNS

C M HUSTWICK

Figure 1.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750
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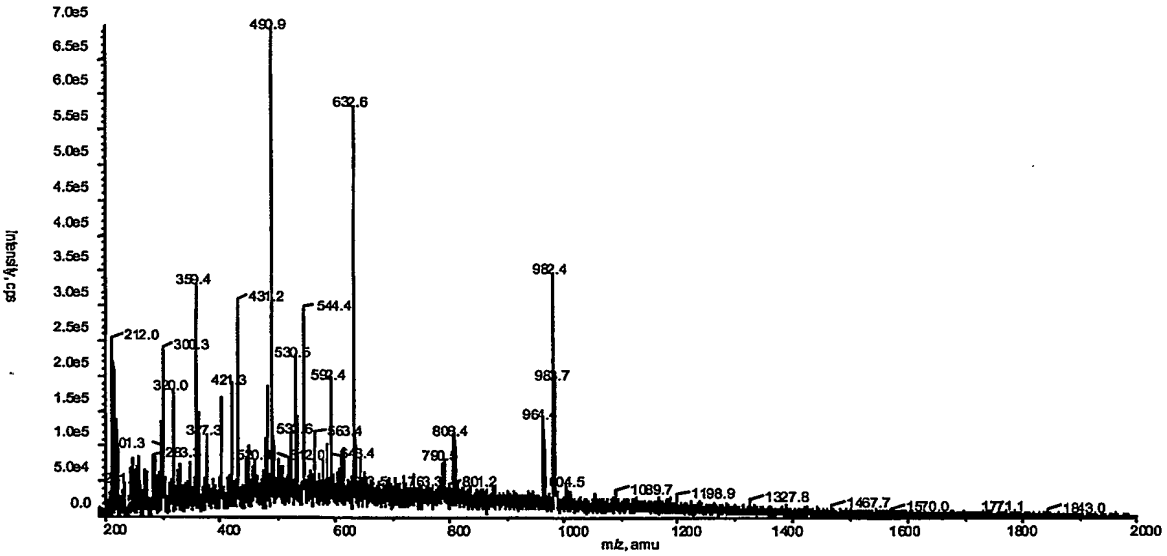
m/z, amu

Figure 2.

A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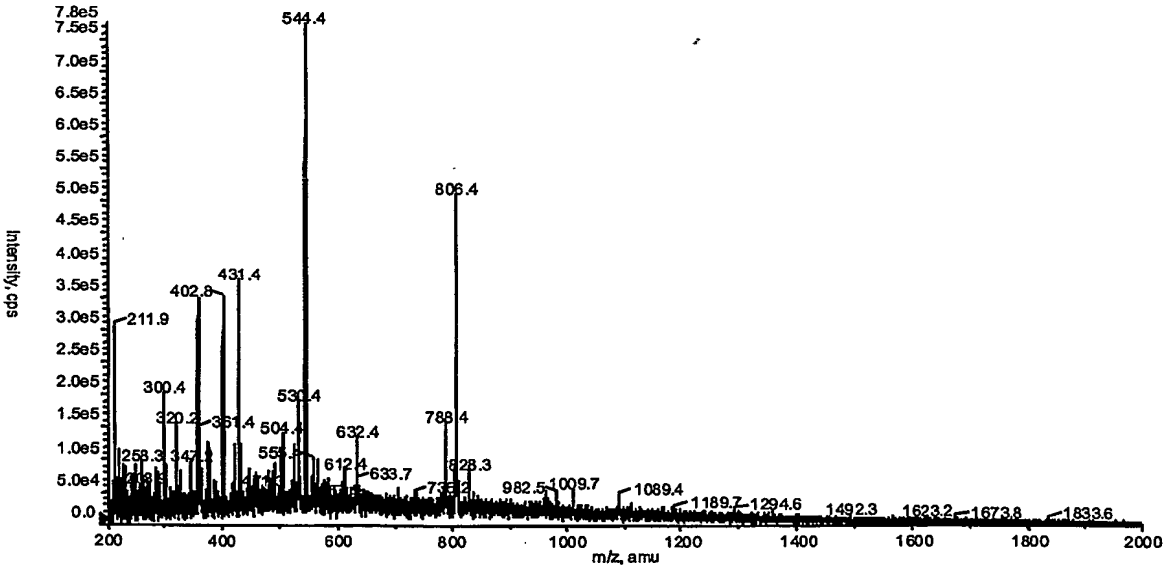
B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1623.2
1673.8
1833.6

Figure 3